EVALUATION OF KINETIC AND THERMODYNAMIC PARAMETERS OF THE NANOIMMUNOSORBENT SIO2-ANTI DICAMBA ANTIBODY-DICAMBA-ALKALINE PHOSPHATASE ANTIGEN SYSTEM IN NANOELISA TECHNIQUE FOR ASSAY OF DICAMBA HERBICIDE IN ENVIRONMENTAL SAMPLES

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Abstract. Homogenous nanoELISA technique based on nanoimmunosorbents: nanoparticles from different materials functionalized with antibodies or antigens can be an alternative for classical ELISA technique, having a high specific area of the nanoimmunosorbents 100 ÷ 200 m2/g. Due to the fact that nanoparticles are in suspension kinetics of the assay is faster, and the stability of the nanoimmunosorbent is given to covalent linking. We used nanoELISA technique to evaluate kinetics parameter like association and dissociation constants between the antibody and homologue antigen, the equilibrium constant and free Gibbs energy of the immune complex. All these parameters are the main characteristics of the nanoELISA technique.

Key words: Dicamba, nanoimmunosorbtent, nanoELISA, polyclonal antiserum, equilibrium constant, Gibbs energy.

1. INTRODUCTION

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is an herbicide used to control broadleaf weeds, being one of the most used pesticide in the world used for agricultural purposes to protect cereals and fruit crops. In combination with a phenoxy herbicide or with other herbicides, dicamba can be used for weed control in range land and other areas: fence rows and roadways. According to the Environmental Protection Agency Pesticide Industry Sales and Usage Report for 2008 and 2012, dicamba is the eighteenth most commonly used conventional pesticide in the agricultural market sector with between 1361 and 3175 tonne only in USA [1]. In soil, dicamba is transformed in 3,6-dichlorosalicylic acid (3,6-DCSA)
by soil bacteria Pseudomonas maltophilia and lacks herbicidal activity [2, 3]. The reported half-life in soil is 1 to 6 weeks [4]. Because of the high solubility in water, residues of this pesticides can contaminate ground water from agricultural fields and surface water also become dangerous for drinking water supplies. As a result dicamba is taken by plants, and can be found in cereals or fruit crops. Livestock can graze dicamba-treated areas and pesticides can be found in their tissues or into milk.Dicamba has toxic effects by ingestion, inhalation or derma exposure, effects on heart, kidney and central nervous system [4, 5]. It is also very irritating and corrosive and can cause severe damages to the eyes [5]. Being used in agriculture and its toxicity requires the necessity to monitor the environmental samples for presence of the pesticide. Different analytical methods have been used to assay this pesticide as high performance liquid chromatography (HPLC) or gas chromatography coupled with mass spectrometry (GC-MC) [3], which are expensive techniques and require long periods of time for analysis. In this case, the enzyme-linked immunosorbent assays (ELISA) is an alternative for detection of dicamba, based on antipesticide antibodies and enzymatic markers for antibody or antigen [6]. In classical ELISA technique antipesticide antibody or antigen is bound to the surface of the well by physical adsorption. The pesticide and enzymatic pesticide markers compete in solution from well with a fixed quantity of bound antibody to the surface of well. However the classical ELISA technique has a lot of disadvantages like desorption of bound reagents from the surface of the well, the antibody-antigen reaction may only take place at the interface between the solid phase and liquid slowing down the rate of the assay, reaching of chemical equilibrium of reaction between antibody and antigen is limited by diffusion of the components from bulk solution to the surface of the wells or tubes and due to limited surface of a well or tubes, high purified and high affinity antibodies are necessary to cover well or tubes.

A nanoELISA technology involving nanoimmunosorbents, nanoparticles functionalized with antibodies or antigens, by covalent bound to the surface of the nanoparticles is proposed. The immune reaction between the anti-pesticide antibody and dicamba-antigen take place in the suspension of the nanoimmunosorbent, and the formed immune complex is found on the surface of the nanoimmunosorbent nanoparticles. Due to their high surface/volume ratio created by the nanoparticles, more binding sites are present for capturing molecules leading to an amplification of the analytical signal [7]. The major advantages of the nanoELISA technique are: the high specific area of the nanoimmunosorbents 100 ÷ 200 m^2/g (2000 cm^2/mg), 1 mg of immunosorbent area is equivalent of twenty ELISA plate (96 wells); time to reach the chemical equilibrium between antibody and antigen is small because the nanoparticles are in suspension (homogenous phase with analyte); molecular diffusion distances are short and the kinetics of the assay are faster, minimizing incubation time necessary to reach the chemical equilibrium; the stability of the nanoimmunosorbents is given due to covalent linking of the antibody on the surface of the nanoparticles. This assay has the potential to be a simple analytical tool for detecting and quantifying dicamba (3,6-dichloro-2-methoxybenzoic acid) levels in
environmental and alimentary samples and potentially a great tool for on-site crop and field monitoring.

2. MATERIALS AND METHODS

3,6-dichloro-2-methoxybenzoic acid (dicamba), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, N-hidroxisuccinimide (NHS), dimethyl formamide (DMF), alkaline phosphatase (ALP), silicon dioxide, (3-aminopropyl) triethoxysilane, glutaraldehyde, Freund’s Adjuvant complete and incomplete and other chemicals were purchased from Sigma-Aldrich (USA).

The anti-dicamba antiserum was obtained by “Cantacuzino” National Institute of Medical-Military Research and Development Bucharest (“Cantacuzino” Institute) by immunizing of the New-Zealand rabbits by intradermal injection on the dorsal side of the neck and trunk in 20 points with dicamba-bovine serum albumin as immunogenic conjugate (Dicamba-BSA). The experiment was conducted in Preclinical Testing Unit, Baneasa Animal Facility, “Cantacuzino” Institute. Fifteen male and female white New Zealand rabbits (“Cantacuzino” Institute) were contained in individual cages with computer controlled humidity (30–70%) and temperature (16–24°C). Animals that were tested were aged between 8–12 months with an average weight of 2.7 kg. Water and food (provided by “Cantacuzino” Institute) were available ad libitum throughout the experiment period. Immunization experiments were undertaken under national and international regulations concerning the protection of animals used for scientific purposes, using a protocol approved by the ethics committee of “Cantacuzino” National Institute of Medical-Military Research and Development certificate no. CE/296/14.09.2018 and advised by Sanitary Veterinary and Food Safety Authority Bucharest (ro. DSVSA Bucharest) authorization number 423/22.10.2018.

Dicamba-alkaline phosphatase (Dic-ALP) was obtained according to the procedures described previously in [8].

Nanoimmunosorbent SiO₂-antipesticide-antibody was also obtained by activation of SiO₂ nanoparticles obtained by initial incubation in 10% HNO₃ for 30 minutes, followed by incubation with 10% (3-aminopropyl)triethoxysilane (APTES) solution for 3 hours at 65°C. Nanoparticles (NPs) were then centrifuged and washed with distilled water followed by addition of 10% glutaraldehyde in 10 mM sodium phosphate buffer for 3 hours at 50°C. Activated NPs were then centrifuged at 1500 xg for 15 minutes, supernatant was removed and then NPs were washed with distilled water and resuspended in 10 mM pH 7.2 sodium phosphate buffer and stored at 4°C.

Nanoimmunosorbent (NIS) antipesticide antibody type was obtained by reaction between activated nanoparticles and specific antipesticide antibodies. Briefly, 1 mL of active NPs suspension was mixed with 1 mL (1 mg/mL) antidicamba antibody solution in sodium phosphate buffer under continuous agitation for 24 h at room
temperature. Nanoimmunosorbents was then centrifuged at 1500 xg for 15 minutes, supernatant was removed and the NIS was resuspended in 10 mM sodium phosphate buffer pH 7.2 and stored at 4°C.

2.1. MATHEMATICAL APPROACH

Reaction between antibody, Ab and antigen, Ag is a bimolecular reversible reaction with formation of the immune complex, Ab-Ag:

\[ Ab + Ag \xrightleftharpoons{K_{+1}}^{K_{-1}} Ab - Ag \]

where: \( K_{+1} \) represents the formation rate constant for the immune complex (forward reaction) and \( K_{-1} \) represents the dissociation rate constant for the complex (reverse reaction).

In this study the antigen is the pesticide Dicamba and the antibody is antidicamba antibody. The mathematical expression of the rate equation for the formation of immune complex:

\[
v = \frac{d[Ab-Dicamba]}{dt} = K_{+1}[Ab][Dicamba] - K_{-1}[Ab-Dicamba]
\]

where: \([Ab]\) is the concentration of antidicamba antibody; \([Dicamba]\) is the concentration of Dicamba antigen; \([Ab-Dicamba]\) is the concentration of the immune complex at one time. If noted \([Ab-Dicamba] = x\), \([Ab] = a\) and \([Dicamba] = p\) relation (1) becomes:

\[
v = \frac{dx}{dt} = K_{+1}ap - K_{-1}x
\]

When establishing the chemical equilibrium the reaction rate becomes zero and so:

\[
K_{+1}a_p x_e = K_{-1}x_e = 0
\]

where \(a_p\), \(p_e\) and \(x_e\) are the concentrations of the three species at chemical equilibrium. If the value of the reverse reaction constant \(K_{-1}\) in relation (3) is replaced, the result is finally given for the value of \(v\):

\[
v = K_{+1}ap - K_{+1} \frac{a_p p_e}{x_e}
\]
Replacing the values of \( a \) and \( p \) according to the initial concentrations of the antibodies, \( a_0 \), of Dicamba pesticide, \( p_0 \) and concentration of immune complex, \( x \):

\[
a = a_0 - x \quad \text{and} \quad p = p_0 - x
\]  

(5)

Reaction rate equation (4) becomes:

\[
v = \frac{dx}{dt} = K_{s1} (a_0 - x)(p_0 - x) - K_{s1} (a_0 - x_e)(p_0 - x_e) \frac{x}{x_e}
\]  

(6)

or

\[
v = \frac{dx}{dt} = \frac{K_{s1}}{x_e} [a_0 p_0 - xx_e][x_e - x]
\]  

(7)

\[
\int \frac{dx}{(a_0 p_0 - xx_e)(x_e - x)} = \frac{K_{s1}}{x_e} \int dt + \alpha
\]  

(8)

By integration is obtained:

\[
\int \frac{dx}{(a_0 p_0 - xx_e)(x_e - x)} = \frac{K_{s1}}{x_e} \int dt + \alpha
\]  

(9)

where: \( \alpha \) coefficient is calculated for the initial conditions for \( t = 0 \); \( a_0 p_0 = a_0 \); \( x_e = \beta \). Thus relation (9) becomes:

\[
\int \frac{dx}{(a_0 - \beta x)(\beta - x)} = \int \frac{A dx}{(a_0 - \beta x)} + \int \frac{B dx}{(B - x)}
\]  

(10)

\[
\frac{A}{a_0 - \beta x} + \frac{B}{\beta - x} = \frac{A(\beta - x) + B(\alpha - \beta x)}{(a_0 - \beta x)(\beta - x)} = 1
\]  

(11)

\[
A\beta - Ax + B\alpha_0 - B\beta x = 1
\]  

(12)

\[
\begin{cases}
A\beta + B\alpha_0 = 1 \\
-Ax - B\beta x = 0 \Rightarrow A = -B\beta
\end{cases}
\]  

(13)

Replacing relation (13) in relation (12) the latter becomes:
By replacing relation (14) with relation (13) the latter becomes:

\[ A = -\frac{\beta}{\alpha_0 - \beta^2} \]  

(15)

Thus, taking into account the relation (15), the relation (10) becomes

\[
\frac{1}{\alpha_0 - \beta^2} \left( -\beta \frac{dx}{\alpha_0 - \beta x} + \frac{dx}{\beta - x} \right) = \\
= \frac{1}{\alpha_0 - \beta^2} \ln(\alpha_0 - \beta x) - \frac{1}{\alpha_0 - \beta^2} \ln(\beta - x) = \\
= \frac{1}{\alpha_0 - \beta^2} \ln \frac{\alpha_0 - \beta x}{\beta - x} = K_{s1}^\alpha + \alpha
\]  

(16)

For \( t = 0 \) and \( x = 0 \) results:

\[ \alpha = \frac{1}{\alpha_0 - \beta^2} \ln \frac{\alpha_0}{\beta} \]  

(17)

Replacing relation (17) in the relation (16), this becomes

\[
\frac{1}{\alpha_0 - \beta^2} \left[ \ln \frac{\alpha_0 - \beta x}{\beta - x} - \ln \frac{\alpha_0}{\beta} \right] = \frac{K_{s1}^\alpha}{\beta} t \]  

(18)

Where from:

\[ K_{s1}^\alpha = \frac{\beta}{\alpha_0 - \beta^2} \frac{1}{t} \left( - \ln \frac{\beta(\alpha_0 - \beta x)}{\alpha_0(\beta - x)} \right) \]  

(19)

In the case of using the enzymatically labelled pesticide, the rate of formation is evaluated from measurements of the enzymatic activity by using a suitable substrate for the enzyme used for marking as follows:

\[
\frac{x}{p_0} = \frac{A^x}{A_0} \quad \text{and} \quad \frac{x}{p_0} = \frac{A^x}{A_0}
\]  

(20)
where: $A'$ represents the enzymatic activity proportional to the concentration of the enzymatic marker bound in the immune complex at chemical equilibrium; $A_s$ represents the enzymatic activity proportional to the concentration of enzymatic marker bound in the immune complex at one point; $A_0$ represents the enzymatic activity proportional to the concentration of enzyme marker introduced into the reaction system; $x_e$ represents the concentration of unlabelled immune complex at chemical equilibrium.

Relation (20) becomes:

$$x_e = \beta = \frac{A'}{A_0} p_0 \quad \text{and} \quad x = \frac{A'}{A_0} p_0$$

or

$$\frac{x}{p_0} = \frac{A'}{A_0} \quad \text{and} \quad \frac{x}{p_0} = \frac{A'}{A_0}$$

Relation (19) becomes

$$K_{s1} = \frac{A_s p_0}{a_0 p_0 - \left( \frac{A'}{A_0} \right)^2} \frac{1}{t} \ln \left( \frac{A'}{A_0} \right) p_0 \left( a_0 p_0 - \frac{A'}{A_0} p_0 \right)$$

$$K_{s1} = \frac{A'}{A_0} \frac{1}{a_0 - \left( \frac{A'}{A_0} \right)^2} \frac{1}{t} \ln \left( \frac{A'}{A_0} \right) a_0 \left( A' - A' \right)$$

For $p_0 \ll a_0$ and $\frac{A'}{A_0} \ll 1$ relation (22) becomes:

$$K_{s1} = \frac{A'}{A_0} \frac{1}{a_0} \frac{1}{t} \ln \frac{A'}{A' - A'}$$

$K$, the equilibrium constant is determined from the relationship (3):

$$K = \frac{K_{s1} x_e}{(a - x_e)(p_0 - x_e)}$$

$$\frac{x_e}{p_0 - x_e} = \frac{A'}{A_0}$$
Replacing relation (24) in relation (25) this becomes:

\[ K_{-1} = K_{s1} a_0 \left( \frac{A_0}{A_e} - 1 \right) \]  (26)

From equation (24) and \((a - x_e) = a_f\), where \(a_f = \) free antibody concentration and \(x^*_e\) represents the concentration of enzymatic labelled immune complex at chemical equilibrium, the equilibrium constant, \(K^*\), in case of using enzymatic labelled antigen becomes:

\[ K^* = \frac{x^*_e}{(a_f - x^*_e)(p_0 - x^*_e)} \]  (27)

For \(K = K^*\),

\[ \frac{x_e}{(a - x_e)(p_0 - x_e)} = \frac{x^*_e}{[a - (x_e + x^*_e)](p_0 - x^*_e)} \]  (28)

For \(x_e \ll p_0\), \(x_e^* \ll p_0\) and \(x_e^* \ll a_f\) equation (28) becomes

\[ \frac{K}{(a - x_e)p_0} = \frac{K^*}{[a - (x_e + x_e^*)]p_0^*} \]  (29)

Or \(\frac{x_e^*}{p_0} = x_e \frac{[a - (x_e + x^*_e)]}{p_0}\), so \(\frac{x_e^*}{p_0} = \frac{x_e}{p_0} \) or \(X_e = p_0 \cdot \frac{x_e^*}{p_0^*}\)

Thus, relation (27) becomes:

\[ K^* = \frac{x_e^*}{a_f \cdot p_0} \]  (30)

3. RESULTS AND DISCUSSION

In case of use ovalbumin-dicamba (OVA-Dic) as antigen having \(M_W \sim 43800\) Da the following antigen inhibition concentrations were used: 0 \(\mu g/0.1\ mL\), 2.5 \(\mu g/0.1\ mL\), 5 \(\mu g/0.1\ mL\), 10 \(\mu g/0.1\ mL\) and 20 \(\mu g/0.1\ mL\). The enzymatic marker used was Dic-ALP, in concentration of 117 \(\text{ng/mL}\) in reaction with para-Nitrophenylphosphate (pNPP) for 30 min. After the centrifugation the absorbance (OD) of the supernatant was measured at 405 nm. The experimental
values are shown in Table 1. Figure 1 shows the inhibition curve of enzymatic labelled antibody-immunosorbent function of concentration of antigen and Figure 2 presents the concentration of immune complex enzymatically unlabelled as function of antigen concentration used as inhibitor.

Table 1
Experimental values of the concentration of the enzyme-labelled immune complex formed on the nanoimmunosorbent depending on the concentrations of OVA-Dic antigen used in the inhibition of the nanoimmunosorbent

<table>
<thead>
<tr>
<th>( p_i ) (µg/0,1mL)</th>
<th>0</th>
<th>2,5</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD</td>
<td>3,019</td>
<td>0,980</td>
<td>0,705</td>
<td>0,614</td>
<td>0,376</td>
</tr>
<tr>
<td>([X^*_e]) (ng/0,1 mL)</td>
<td>4,760</td>
<td>1,545</td>
<td>1,110</td>
<td>0,960</td>
<td>0,560</td>
</tr>
<tr>
<td>([X_e]) (µg/mL)</td>
<td>0</td>
<td>0,033</td>
<td>0,047</td>
<td>0,082</td>
<td>0,096</td>
</tr>
</tbody>
</table>

Fig. 1 – Inhibition curve of enzymatic labelled antibody-immunosorbent with concentration of antigen.

Fig. 2 – Concentration of immune complex enzymatically unlabelled as function of antigen concentration used as inhibitor.
3.1. CALCULATION OF THE EQUILIBRIUM CONSTANT

From the reaction of antibody with labelled antigen and equations (28–30), concentrations of antibody and labelled antigen are:

\[ \text{[Ab]} = 2.46 \cdot 10^{-8} \text{ mol/L and [Ag]*} = 1.36 \cdot 10^{-8} \text{ mol/L} \]

For \( X_v^* = 4.76 \text{ ng/tube} \), \( M^{OVA-Dic}_{w} = 43800 \text{ Da} \) and \( M^{Dic-ALP}_{w} = 86000 \text{ Da} \) one obtains: \( X_v^* = 5.5 \cdot 10^{-10} \text{ mol/L} \) and \( K = 1.65 \cdot 10^6 \text{ L/mol} \).

Experimental values for calculation the formation rate constant for the immune complex, \( K_{+1} \) are presented in Table 2.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>OD</th>
<th>( A'_0 ) (ng)</th>
<th>( A'_0 - A' ) (t)</th>
<th>( \frac{A'_0}{A'_0 - A' \text{(t)}} )</th>
<th>( \text{ln} \frac{A'_0}{A'_0 - A' \text{(t)}} )</th>
<th>( K_{+1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.17</td>
<td>1.85</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>0.96</td>
<td>1.51</td>
<td>0.34</td>
<td>5.44</td>
<td>1.69</td>
<td>2.16 \cdot 10^5</td>
</tr>
<tr>
<td>10</td>
<td>0.765</td>
<td>1.21</td>
<td>0.64</td>
<td>2.89</td>
<td>1.06</td>
<td>0.70 \cdot 10^5</td>
</tr>
</tbody>
</table>

Calculated medium values are \( K_{+1} = (1.43 \pm 0.13) \cdot 10^3 \text{ L/mol \cdot min} \)

\( K_{+1} = 8.67 \cdot 10^{-2} \text{ min}^{-1} \)

Gibbs Energy: \( \Delta G = RT \ln K = 7494 \text{ J/mol} \)

Fig. 4 – Inhibition curve of antibody-type nanoimmunosorbent depending on antigen concentration.
In Figure 4 is presented the inhibition curve of antibody-type nanoimmunosorbent depending on antigen concentration presented as the effect of the concentration of OVA-Dic inhibition antigen (unlabelled enzymatically) to the ratio between the OD of the inhibited sample and the OD of the uninhibited sample (100% enzymatically labelled sample).

4. CONCLUSIONS

In this work we used nanoimmunorients based on SiO\textsubscript{2} nanoparticles functionalized with polyclonal anti-dicamba antibody in reaction with dicamba antigen. Two antigens, ovalbumin-dicamba and dicamba-alkaline phosphatase as enzymatic labelled antigen were used to evaluate the association rate constant \( K_+ = (1.43 \pm 0.13) \times 10^5 \text{ L/mol} \cdot \text{min} \), the dissociation rate constant \( K_- = 8.67 \times 10^{-2} \text{ min}^{-1} \) and the equilibrium constant \( K = 1.65 \times 10^6 \text{ L/mol} \) of the immune system. Also it was calculated the free energy \( \Delta G = 7494 \text{ J/mol} \) and the time to reach the chemical equilibrium 10–15 minutes. All these parameters are important characteristics of the nanoELISA technique for assay of dicamba pesticides in environmental factors.

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